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EFFECT OF DEGRADATION KINETICS ON THE MICROSTRUCTURE OF ANAEROBIC BIOGRANULES

Herbert H. P. Fang*, Ho-Kwong Chui* and Yu-You Li**

** Environmental Research Centre, Department of Civil and Structural Engineering, The University of Hong Kong, Pokfulam Road, Hong Kong*
*** Department of Civil Engineering, Faculty of Engineering, Tohoku University, Aoba, Sendai 980, Japan*

ABSTRACT

The microstructure of anaerobic biogranules treating a wide variety of wastewaters was investigated using light and scanning electron microscopies. Biogranules were sampled from upflow anaerobic sludge blanket (UASB) reactors treating wastewater individually containing formate, acetate, propionate, butyrate, peptone, sucrose, starch, benzoate, brewery and monosodium glutamate. Results indicated that the microstructure of the biogranules was strongly dependent on the degradation kinetics of substrates. Anaerobic degradation is a multi-step process, involving fermentation/acidogenesis, acetogenesis and methanogenesis. For substrates, such as carbohydrates, of which the initial step of degradation was considerably faster than the subsequent degradation of intermediates, biogranules developed a layered microstructure. On the other hand, for substrates, such as proteins, of which the initial step of degradation was rate-limiting, a uniform microstructure would be developed. These findings are of significance for the development of kinetic models for biogranule and biofilm.

KEYWORDS

Anaerobic; biofilm; biogranule; degradation; kinetics; microstructure; population; rate; substrate; UASB.

INTRODUCTION

Anaerobic filter (Young and McCarty, 1969) and upflow anaerobic sludge blanket (UASB) reactors (Lettinga *et al.*, 1980; Lettinga and Hulshoff Pol, 1991) have become widely accepted in recent years for the treatment of industrial wastewater containing concentrated organic pollutants. Both reactors are capable of retaining large amounts of biomass, which form biofilms on support media in the former and biogranules in the latter reactors. Recent studies have shown that UASB biogranules not only exhibited high settleability and methanogenic activity (Fang and Chui, 1993), but also were capable of degrading aromatic pollutants, such as benzoate (Li *et al.*, in press) and phenol (Fang *et al.*, 1986). Since many complex aromatic compounds degrade via benzoate and phenol, these findings suggest that anaerobic technology could likely be extended to the treatment of wastewater from many chemical industries.

The development of mathematical models for anaerobic reactors (McCarty and Mosey, 1991) has been severely hindered by the lack of understanding on the microstructure of biofilm and biogranule, and the influence of biodegradation kinetics (Dolfing *et al.*, 1985; Dubourguier *et al.*, 1988). Early investigations of

the microstructure of the biogranules were contradictory and inconclusive. MacLeod, *et al.* (1990) proposed a three-layered microstructure for the sucrose-degrading biogranules, which was supported by Guiot *et al.* (1992) in their study of glucose-degrading biogranules. Grotenhuis *et al.* (1991), on the other hand, found that there was no evidence of a layered microstructure for biogranules treating propionate, ethanol, and sugar refinery wastewaters. Fang *et al.* (1994a) also found that peptone-degrading biogranules did not exhibit a layered structure; instead, they were densely packed with intertwined bacteria of different morphologies throughout the granule cross-section.

Anaerobic degradation is a multi-step process (Gujer and Zehnder, 1983; Thiele and Zeikus, 1988). Complex organic substrates, such as proteins and carbohydrates, are first hydrolyzed by enzymes forming soluble amino acids and sugars, which are then degraded by acidogenic bacteria into volatile fatty acids (VFA); benzoate, on the other hand, is the key intermediate in the degradation of aromatic compounds (Knoll and Winter, 1989). These intermediate acids are further degraded by acetogenic bacteria forming acetate, formate, carbon dioxide and hydrogen. This final group of intermediates are ultimately converted to methane by the methanogenic bacteria. In the degradation of intermediates, including benzoate, propionate and butyrate, syntrophic associations between the hydrogen-producing acetogens and hydrogen-consuming methanogens are often required.

A series of studies were recently conducted to investigate the performance of UASB reactor in treating wastewaters individually containing several model substrates and key intermediates (Fang and Chui, 1993; Chui *et al.*, 1994; Fang and Kwong, 1994; Fang *et al.*, 1994a, 1995a,b; Li *et al.*, in press). The individual microstructures of several biogranules were reported, along discussion of many other parameters, such as COD (chemical oxygen demand) removal efficiency, maximum SSUR (specific substrate utilization rate) of biogranules, sludge yield, etc.. This paper is, however, to develop general models of biogranule microstructure to correlate them to the degradation kinetics of the organic substrates, based on information obtained in these prior studies.

MATERIALS AND METHODS

Biogranules treating brewery and monosodium glutamate effluents were obtained from full-scale UASB reactors. Others biogranules were sampled from a number of laboratory UASB reactors (2.8 and 8.5 litres in size) after being operated at the steady-state COD loading rate of 10 g/l d for over 5 months. Individual substrates for the laboratory reactors included formate, acetate, propionate, butyrate, sucrose, starch, peptone, and benzoate. The sampled biogranules were examined for the distribution of methanogens using light microscopy (Chui and Fang, 1994), and for the bacterial morphology using scanning electron microscopy (SEM). Details of the reactor conditions, biogranule characteristics and the preparation for microscopic investigations are available elsewhere (Fang and Chui, 1993; Chui *et al.*, 1994).

RESULTS

The microstructure of the biogranules could be classified into two main categories: layered and uniform. The former could be further subdivided into two-layered and three-layered, whereas the latter could be either simple or complex with intertwined bacteria of various morphologies.

Biogranules with layered microstructure

Biogranules having layered microstructure included those utilizing butyrate, benzoate, sucrose, starch and carbohydrates in brewery wastewater as organic substrates. Among them, butyrate- and benzoate-degrading biogranules exhibited a two-layered microstructure, while the three carbohydrate-degrading ones exhibited a three-layered microstructure. The outer layer of the butyrate-degrading biogranules (Fang *et al.*, 1995a) had a thickness of 20–40 μm. Under epi-fluorescent excitation at 350 nm and 420 nm (Doddema and Vogels, 1978), this layer emitted intense fluorescence (Fig. 1a) due to the presence of hydrogenotrophic methanogens. SEM micrographs showed that this layer was densely packed with microcolonies composed of two species of bacteria which appeared to be juxtaposed for syntrophic association. The interior was mainly

composed of uniformly distributed *Methanothrix*-like bacteria (Fig. 1b), which emitted much dimmer fluorescence (Zehnder *et al.*, 1980). Similar to those degraded butyrate, benzoate-degrading biogranules (Li *et al.*, in press) also exhibited a two-layered microstructure, in which the outer layer was composed of an abundance of *Syntrophus buswellii*-like bacteria (Tarvin and Buswell, 1934), which converted benzoate into acetate, whilst the interior was mainly composed of *Methanothrix*-like filaments.

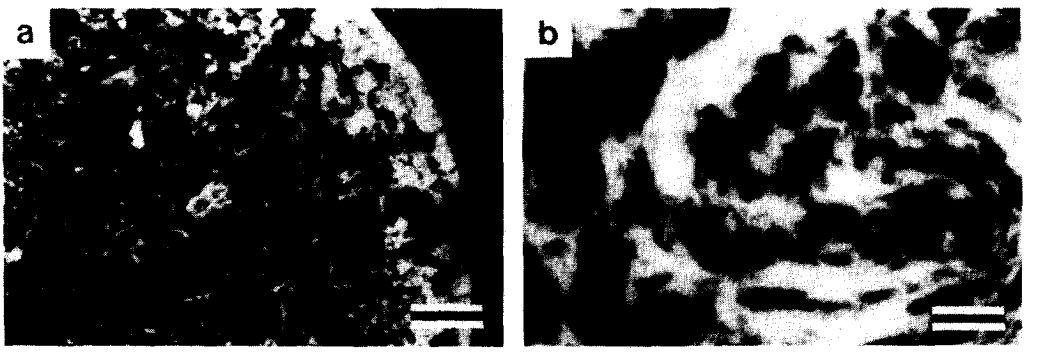

Figure 1. Butyrate-degrading biogranules with (a) an outer layer comprising hydrogenotrophic methanogens (bar = 100 μm), and (b) a centre comprising *Methanothrix*-like filaments (bar = 10 μm).

For the three-layered carbohydrate-degrading biogranules, the outer layer was composed of a variety of bacteria with few fluorescence-emitting methanogens. Its middle layer and centre resembled the outer layer and the centre core of the two-layered butyrate-degrading biogranules. Figure 2a illustrates a sucrose-degrading biogranule (Fang and Chui, 1993) exhibiting a dense skin and an interior packed with *Methanothrix*-like bacteria under light microscope. Under epi-fluorescent excitation (Fig. 2b), the skin consisted of two layers: an outer layer emitting little fluorescence and a second layer comprising fluorescent methanogens of diverse morphologies, including cocci, bacilli and some filaments.

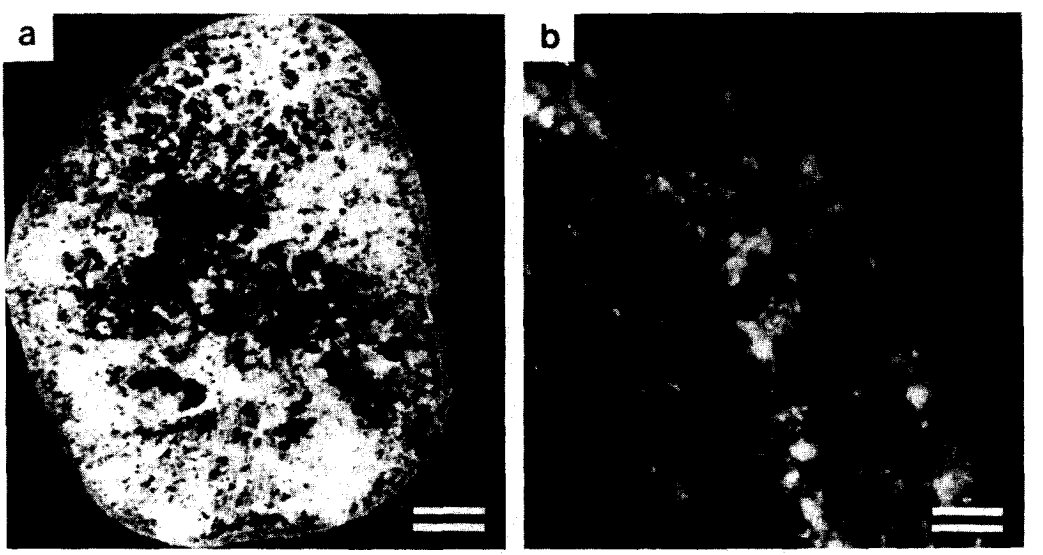

Figure 2. Sucrose-degrading biogranules with (a) an interior packed with *Methanothrix*-like filaments (bar = 100 μm) and a densely packed skin (b), which comprises an outer and a middle layers (bar = 10 μm).

For biogranules treating complex carbohydrates, the outer and the middle layers became thicker and more complex. Biogranules treating starch as the sole substrate had a well-defined three-layered microstructure (Fang and Kwong, 1994). The 10–20 μm outer layer was mainly composed of streptococci, which were responsible for the initial hydrolysis of starch, while the 100–200 μm middle layer was composed of long unidentified filaments and syntrophic microcolonies. The centre core was composed of filamentous *Methanothrix*-like bacteria.

Biogranules treating brewery wastewater containing carbohydrates as substrate had a size of 2–4 mm; their outer and middle layers were about 100 μm in thickness (Fang *et al.*, 1994b). The majority of bacteria in the outer layer were acidogens, as illustrated by its low fluorescent intensity under excitation, and some scattered *Methanothrix*-like colonies. The middle layer was, on the other hand, predominantly syntrophic microcolonies comprising hydrogenotrophic methanogens with high fluorescent intensity. The centre core was densely packed with short-rod-shaped *Methanothrix*-like bacteria, different from those in the sucrose- and starch-degrading biogranules.

Biogranules with uniform microstructure

Biogranules degrading formate, acetate, propionate, peptone, and MSG wastewater had a uniform microstructure. Conversions of formate and acetate into methane are one-step processes. Biogranules degrading formate and acetate were small, and each had a simple uniform microstructure comprising a predominant species of methanogen. Formate-degrading biogranules (Chui *et al.*, 1994) were of irregular shape with sizes less than 0.5 mm. As illustrated in Fig. 3, they were mainly composed of rod-shaped filamentous *Methanobacterium formicicum*-like bacteria (Mah and Smith, 1981). Acetate-degrading biogranules were also small (less than 1 mm in size); they were predominantly composed of *Methanothrix*-like filaments and scattered clusters of *Methanosarcina*-like cysts (Mah and Smith, 1981).

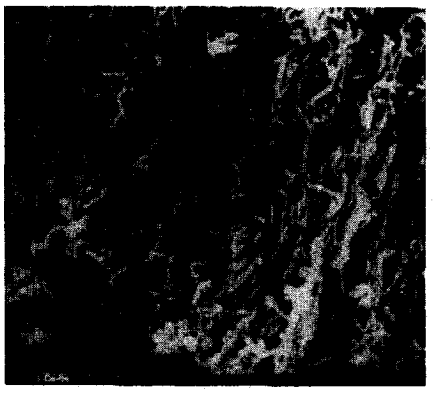

Figure 3. Formate-degrading biogranules comprising *Methanobacterium formicicum*-like bacteria (bar = 10μm).

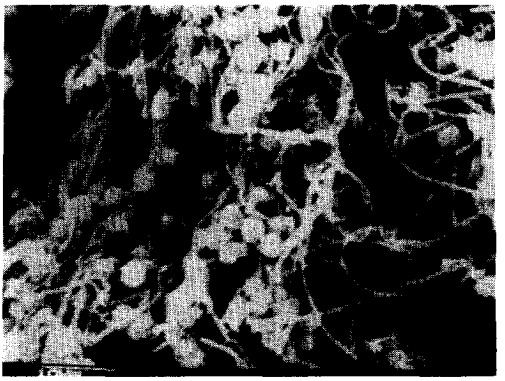

Figure 4: Peptone-degrading biogranules comprising intertwined bacteria of different morphologies (bar = 10 μm).

On the other hand, conversions of propionate, peptone and MSG are multi-step processes. Typical propionate-degrading biogranules were 1–2 mm in size and had a uniform microstructure comprising scattered *Methanothrix*-like microcolonies and juxtapositioned syntrophic microcolonies (Fang *et al.*, 1995b). Peptone-degrading biogranules (Fang *et al.*, 1994a), on the other hand, had a porous surface, but a densely packed interior with intertwined bacteria of different morphologies (Fig. 4). Although there was no predominant species of bacteria, microcolonies comprising *Methanothrix*- and *Methanosarcina*-like bacteria and those comprising juxtapositioned syntrophic bacteria were found scattered in the interior of the

biogranules. Similarly, biogranules degrading MSG also developed a uniform, but complex microstructure (Fang *et al.*, 1994b).

DISCUSSION

Formation of layered microstructure

During anaerobic degradation, complex substrates are converted by fermentative/acidogenic bacteria into VFA, which were further converted by acetogens forming acetate, prior to the final formation of methane by methanogens. The rate of each step depends on the concentration of reactants, bacterial species, and a number of parameters, such as pH and temperature. In the cases that the initial step of substrate degradation is significantly faster than the subsequent degradation of intermediates, most of the substrate are consumed by bacteria near the biogranule surface. The concentration of intermediates would build up, causing them to diffuse toward the biogranule interior, due to concentration gradient, for further degradation. As a consequence, the biogranule develops a layered structure, where the outer layer is mainly responsible for the rapid initial step of substrate degradation and the inner layer(s) for the subsequent degradation of intermediates (Fig. 5a).

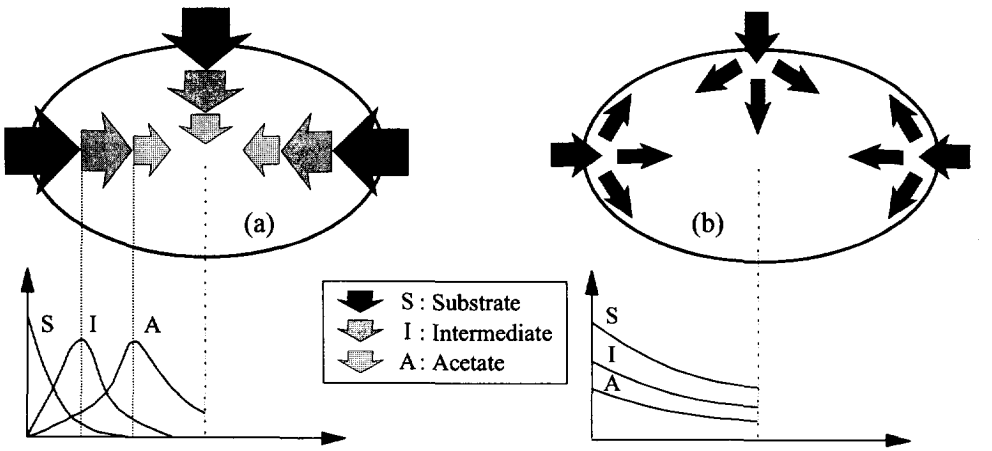

Figure 5. Diffusion and concentration profiles of biogranules with (a) layered microstructure, and (b) uniform microstructure.

Table 1 summarizes the maximum specific substrate utilization rate of enrichment culture at 35°C for a number of substrates. Propionate was converted to acetate at the rate similar to the subsequent conversion of acetate to methane. Acetogenesis of butyrate was however twice as fast. On the other hand, carbohydrates, including glucose, sucrose and starch, were converted to VFA at rates about 5–10 times faster than acetotrophic methanogenesis.

Fang *et al.* (1995a) studied the degradation of butyrate in a UASB reactor and confirmed that acetotrophic methanogenesis was the rate-limiting step. This was also reflected on the two-layered microstructure of butyrate-degrading biogranules. Most of butyrate was readily converted to acetate in the outer layer, while the acetate produced were allowed to diffuse to the interior before methanogenesis taking place. Li *et al.* (in press), based on some intriguing observations, suggested that benzoate degradation is a two-step process. Bacteria, such as *Syntrophus buswellii,* rapidly converted benzoate inside their cells directly into acetate, which was subsequently converted to methane by methanogens at a slower rate. Like those degrading butyrate, benzoate-degrading granules also exhibited a two-layered microstructure.

Table 1. Maximum specific substrate utilization rate at 35°C

Reaction	Substrate	Maximum SSUR (g-COD/g-VSS·day)	Reference
Acetotrophic methanogenesis	acetate	4.8	Zehnder, *et al.* (1980)
Acetotrophic methanogenesis	acetate	6.8 - 13.1	Lawrence and McCarty (1969)
Acetotrophic methanogenesis	acetate	4.4 - 11.6	Noike, *et al.* (1985)
Acetogenesis	propionate	6.2	Gujer and Zehnder (1983)
Acetogenesis	propionate	8.2 - 12.2	Lawrence and McCarty (1969)
Acetogenesis	butyrate	16.6	Lawrence and McCarty (1969)
Fermentation of carbohydrates	glucose	72	Zeotemeyer, *et al.* (1982)
Fermentation of carbohydrates	sucrose	71	Noike, *et al.* (1985)
Fermentation of carbohydrates	starch	40	Noike, *et al.* (1985)

In the degradation of carbohydrates, the rate-limiting step is also the acetotrophic methanogenesis (Henze and Harremoes, 1983), as shown in Table 1. The initial acidogenesis of carbohydrates took place rapidly at the outer layer; the intermediate VFA, including butyrate and propionate, degraded at a lower rate, and diffused to the interior of the biogranule. These intermediates were then degraded by acetogens in the middle layer forming acetate. Since the methanogenesis step was rate-limiting, the acetate diffused toward the biogranule centre where methanogenesis took place. As a result, carbohydrate-degrading biogranules developed a three-layered microstructure.

The thickness of the outer layer, where fermentation and acidogenesis took place, was dependent on the complexity of the carbohydrate. The trend seemed to be the more complex the substrate, the thicker the outer layer. Among the three types of carbohydrate-degrading biogranules, those treating brewery wastewater had the thickest outer layer, followed by the starch-degrading ones, and the sucrose-degrading biogranule had the thinnest outer layer.

Formation of uniform microstructure

In the degradation of formate and acetate, the substrate was converted into methane in a one-step process, each by a predominant species of methanogen, the biogranules resulting in a simple, uniform microstructure. In addition, these two biogranules were the smallest in size among all the biogranules examined.

For biogranules degrading substrates of which the initial degradation was slow relative to the subsequent degradation of intermediates, a considerable fraction of substrate would diffuse toward the interior before being degraded. Substrate concentration became quite uniform over the biogranule cross-section; as a result, biogranules developed a uniform microstructure with even distribution of all sorts of bacteria involved at various stages of degradation (Fig. 5b). This was the case for the propionate-, peptone- and MSG-degrading biogranules. For these substrates, the initial steps of degradation, including acetogenesis of propionate (Fang, 1995b), hydrolysis of protein (Gujer and Zehnder, 1983) and acidogenesis of glutamate, were rate-limiting.

Relationship of degradation kinetics and microstructure

The biogranule microstructure is dependent of the complexity of the substrate and rate of the initial degradation relative to the subsequent degradation of intermediates. As illustrated in Fig. 6, biogranules degrading complex substrates with rapid initial degradation exhibit multi-layered microstructure; those treating substrates of which the initial degradation is slow relative to subsequent degradation of intermediates, exhibit a complex, but uniform microstructure. On the other hand, biogranules degrading

simple substrates with rapid initial degradation exhibit a two-layered microstructure, while those with slow initial degradation rate relative to the degradation of intermediates exhibit a simple, uniform microstructure.

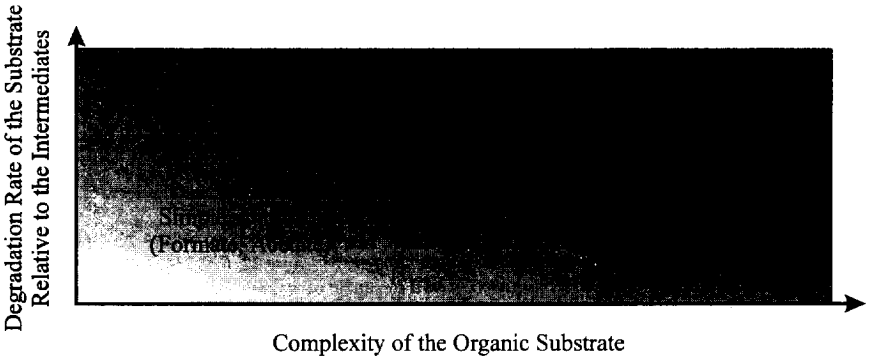

Figure 6. Relationship of microstructure of biogranules and degradation kinetics and substrate complexity.

CONCLUSION

The microstructure of the biogranules could be classified into two main categories: layered and uniform. The former could be further subdivided into two-layered and three-layered, whereas the latter could be either simple or complex with intertwined bacteria of various morphologies. The microstructure is strongly dependent on the degradation kinetics of the substrate. Findings from this study should be valuable for the development of mathematical models of anaerobic biogranules and biofilms.

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